REPORT DOCUMENTATION PAGE	Form Approved OMB NO. 0704-0188

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE 1/23/06	3. REPORT TYPE AND DATES COVERED 4/1/01 - 06/01/05

4. TITLE AND SUBTITLE	5. FUNDING NUMBERS
The Adaptive Response to Intestinal Oxidative Stress in Mammalian Hibernation	DAAD19-01-1-0455
6. AUTHOR(S) Hannah V. Carey, Ph.D.	

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER DUNNS number:
University of Wisconsin-Madison 2015 Linden Drive, Madison, WI 53706	161202122

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSORING / MONITORING AGENCY REPORT NUMBER
U. S. Army Research Office P.O. Box 12211 Research Triangle Park, NC 27709-2211	42419.2-LS

11. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

12 a. DISTRIBUTION / AVAILABILITY STATEMENT	12 b. DISTRIBUTION CODE
Approved for public release; distribution unlimited.	

13. ABSTRACT . The goal of this project is demonstrate how mammalian hibernators utilize the physiologic consequences of metabolic depression, which include changes in mitochondrial function, low body temperatures (T_b) and reduced blood flow, to activate cellular signaling pathways that minimize oxidative damage to sensitive tissues during torpor-arousal cycles. Specific Aim 1 examines oxidative stress to the gut of ground squirrels during the seasonal cycle. Specific Aim 2 examines consequences of intestinal oxidative stress during hibernation including seasonal changes in NF-κB activation in intestine, seasonal changes in the intestinal mucosal immune system and the effect of hibernation on enterocyte apoptosis and cell cycle regulators. We have now developed two models of intestinal ischemia-reperfusion (I/R) in rats and ground squirrels, and preliminary studies strongly suggest that the hibernation phenotype confers protection against gut-induced trauma. Mechanisms of protection include maintenance of intestinal absorptive and barrier function and suppression of the I/R– induced inflammatory cascade (e.g., neutrophil activation, cytokine release). Importantly, these protective effects are not dependant upon hypothermia or hypometabolism, as they are present in fully aroused hibernators. Our findings provide insight into the dynamic nature of the hibernating phenotype in terms of protection against oxidative stress and suppression of pro-inflammatory processes. We anticipate that further understanding of this naturally-induced change in a mammalian model for extreme physiologic change can lead to novel approaches to protect and preconditioning humans against traumatic events.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Hibernation; oxidative stress; antioxidant defenses; redox balance; intestine; immune system	9
	16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT	18. SECURITY CLASSIFICATION ON THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	**UNCLASSIFIED**	**UNCLASSIFIED**	**UL**

THE ADAPTIVE RESPONSE TO INTESTINAL OXIDATIVE STRESS IN MAMMALIAN HIBERNATION
Department of Defense, Army Research Office DAAD19-01-1-0455

(1) List of all papers published or in progress since start of award

van Breukelen, F. and H.V. Carey. 2002. Ubiquitin conjugate dynamics in the gut and liver of hibernating ground squirrels. *J. Comparative Physiology B* 172: 269-273.

Balslev-Clausen, A., J. M. McCarthy and H. V. Carey. 2003. Hibernation reduces pancreatic amylase levels in ground squirrels. *Comp. Biochem. Physiol. A* 134:573 – 578.

Carey, H.V., C.A. Rhoads and T.Y. Aw. 2003. Hibernation induces glutathione redox imbalance in ground squirrel intestine. *J. Comp. Physiol. B* 173:269-276.

Carey, H.V., M.T. Andrews and S.L. Martin. 2003. Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. *Physiological Reviews* 83: 1153-1181.

Carey, H.V. 2005. Gastrointestinal responses to fasting in mammals: lessons from hibernators. pp 229-254 In: Physiological and Ecological Adaptations to Feeding in Vertebrates (Eds. J.M. Starck and T. Wang). Science Publishers Inc., New Delhi

Fleck, C. and **H.V. Carey**. 2005. Modulation of apoptotic pathways in intestinal mucosa during hibernation. *Amer. J. Physiol. Regul. Integr. Comp. Physiol* 289: R586-R595.

Fleck, C.C and H.V. Carey. . Hibernation confers resistance to organ damage following intestinal ischemia-reperfusion. *To be submitted by June, 2006*

Fleck, C.C and H.V. Carey. Seasonal changes in the mucosal immune system in the hibernating rodent, Spermophilus tridecemlineatus. *To be submitted by June, 2006*

Papers presented at meetings:

Carey, H.V. and C.C. Fleck. 2001. Increased recruitment of intraepithelial lymphocytes into small intestinal mucosa during metabolic depression in ground squirrels. *Gastroenterology* 120:A319.

Fleck, C.C. and H.V. Carey. 2001. Activation of NF-κB in intestinal mucosa of hibernating ground squirrels involves activation of the IKK/IκB pathway. *FASEB J.* 15: A818.

Fleck, C.C. and H.V. Carey. 2002. Changes in the apoptotic pathway in intestinal epithelial cells during hibernation. *The Physiologist* 45:313

Fleck, C.C and H.V. Carey. 2003. Expression of anti-apoptotic pathways in intestinal epithelial cells during hibernation. *FASEB J.* 17: A39.

Carey, H.V. Speaker and co-chair for symposium on "Cellular and Molecular Responses to Depressed Metabolism and Low Temperature". My presentation was entitled: "Stress-induced signaling pathways associated with depressed metabolism and low temperature" Meeting: American Physiological Society Intersociety Conference on Comparative Physiology: Evolution, Adaptation and Application (San Diego, Aug 24-28, 2002)

Fleck, C.C., Lindell, S.L., Luterbach, K.J., Mangino, M.J. and H.V. Carey. 2004. Hibernation confers resistance to intestinal ischemia/reperfusion injury. Poster presentation at Life in the Cold: Evolution, Mechanisms, Adaptation and Application. Twelfth International Hibernation Symposium, Seward, AK.

Clayton, M.K., Fleck, C.C. and H.V. Carey. 2004. Analysis of torpor-arousal cycles in the 13-lined ground squirrel. Poster presentation at Life in the Cold: Evolution, Mechanisms, Adaptation and Application. Twelfth International Hibernation Symposium, Seward, AK.

Fleck, C.C and H.V. Carey. 2005. Hibernation alters mucosal cytokine expression in ground squirrel intestine. Experimental Biology 2005 Abstract #406.13. [accessed at http://select.biosis.org/faseb/eb2005_data/FASEB001431.html].
Fleck, C.C and H.V. Carey. 2005. Hibernation confers resistance to organ damage following intestinal ischemia-reperfusion. *Gastroenterology* 128:A119.

(2) Scientific Personnel:

2001-2002:
Courtney Fleck, Ph.D. student. will complete Ph.D. in June, 2006. Stipend was not supported by grant but all research carried out by Ms. Fleck for her Ph.D. (supplies, equipment, etc.) is supported by this ARO award.
Melissa Lefton, Undergraduate student, worked as student hourly
Timothy Piazza, Research Specialist

2002-2003:
Courtney Fleck, Ph.D. student.
Andreas Balslev-Clausen, visiting medical student from Univ of Copenhagen. Was supported by a Fulbright scholarship to work in our laboratory for 9 months in 2002 and participated in the ARO-funded project.
Julia McCarthy, undergraduate student, worked initially for academic credit, then for hourly wage on ARO-funded project
Jessica Reimer, Research Specialist; replaced by Katie Luterbach in July, 2003
Katie Luterbach, B.S., Research Specialist on project since July, 2003

2003-2004:
Courtney Fleck, Ph.D. student.
Sean Jungbluth, undergraduate student, worked on project for academic credit.

(3) Report of inventions: N/A.

(4) Scientific Progress:
I. The following is a summary of the work proposed for this project and an update of the status of each section of the project as of June, 2005.

General Project Goals:

The long-term goal of this research program is to identify signaling pathways that link external stressors to cellular and molecular responses that provide tolerance to severe stress. The project uses ground squirrels as a natural model system to identify stress-induced defensive mechanisms in mammals. As a hibernating species, ground squirrels undergo each year profound changes in physiology that would be considered highly stressful for non-hibernating species such as humans. The specific goal of this research project is to test the hypothesis that the intestine is vulnerable to oxidative stress during the hibernation season, and as a consequence, activates defensive mechanisms that promote the maintenance of intestinal structure and function. A unifying theme of this research is that hibernators exploit the physiologic consequences of metabolic depression and low temperatures to induce cellular signaling pathways that minimize oxidative damage to sensitive tissues like the gut.

Progress on Original Aims 1 and 2 of Project

Aim 1: Effect of hibernation on intestinal lipid peroxidation, redox status and pro- and anti-oxidant enzymes.

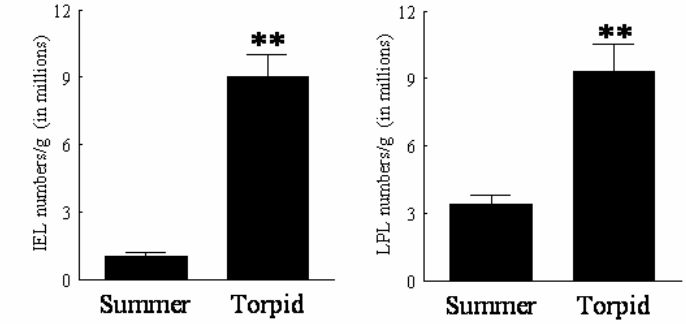
a) Determination of oxidized lipids in intestinal mucosa: Due to technical problems, samples were unable to be assayed by collaborator Dr. Craig Frank for levels of oxidized lipids. We hope to complete these analyses at a later time.
b) Characterize seasonal changes in intestinal glutathione (GSH) redox system: The experiments for this portion of the project have been completed and a manuscript was published (Carey, H.V., C.A. Rhoads and T.Y. Aw. 2003. Hibernation induces glutathione redox imbalance in ground squirrel intestine. *J. Comp. Physiol. B* 173:269-276).

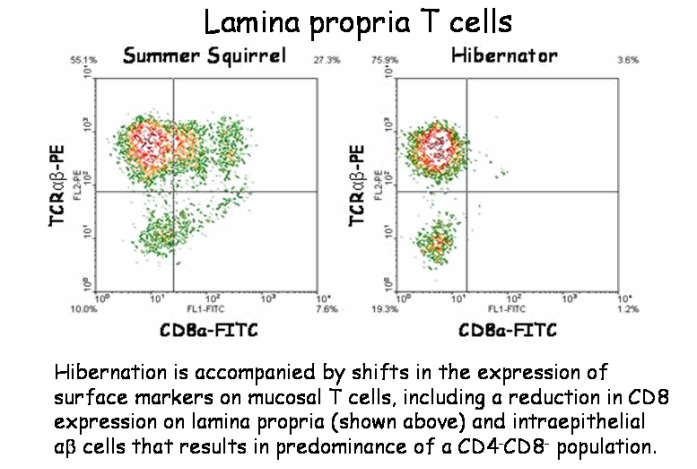
Aim 2. Consequences of oxidative stress in the intestine during hibernation.

a) Determine seasonal changes in NF-κB activation and identify activated cells: During the preparation of this manuscript we realized some experiments still needed to be completed on this aim, thus this line of research is still under investigation.
b) Determine seasonal changes in the intestinal mucosal immune system.

These studies, that use immunohistochemistry, flow cytometry and ELISA assays are nearly complete. The figures below show the key results.

Hibernation is associated with increased numbers of mucosal lymphocytes, including intraepithelial (IEL) and lamina propria lymphocytes (LPL).



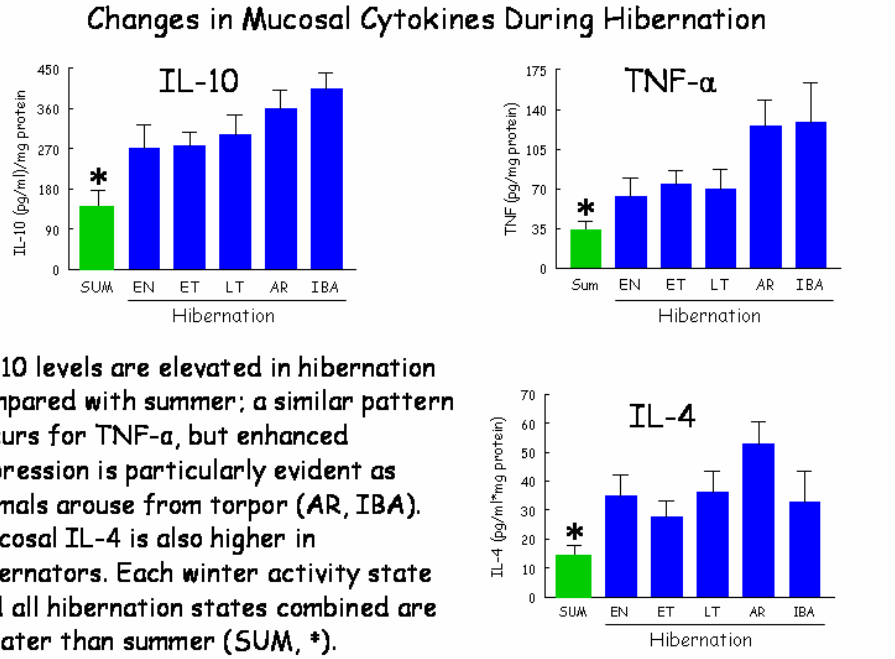


Hibernation is accompanied by shifts in the expression of surface markers on mucosal T cells, including a reduction in CD8 expression on lamina propria (shown above) and intraepithelial αβ cells that results in predominance of a $CD4^{-}CD8^{-}$ population.

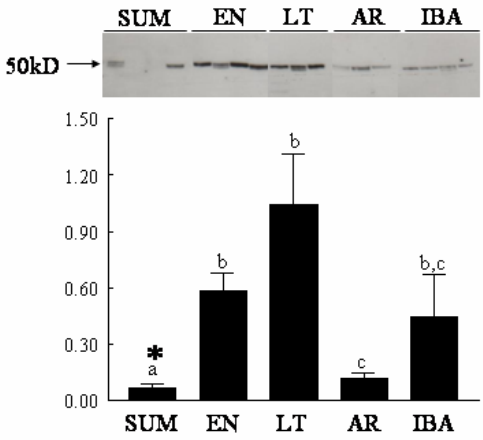
Analyses still in progress have revealed significant changes in the types of surface phenotypes found on these lymphocytes between hibernating and summer states. Shown on left is one example.

Changes in levels of mucosal cytokines induced by the hibernation phenotype. These experiments were designed to determine whether and how hibernators modulate their mucosal immune defense systems to minimize damage to the gut during the hibernation season by expression of relevant mucosal cytokines. We identified seasonal changes in the following

mucosal cytokines: IL-10, TNF-α, interferon-γ, IL-4, IL-6, with some examples shown below. We believe the sustained, high levels of IL-10, an anti-inflammatory cytokine, may be a response to, and help protect the gut against elevations in pro-inflammatory cytokines during the arousal process, such as TNF-α and interferon-γ, which both show elevated levels during the hibernation season.



Seasonal changes in secretory IgA. The elevated IL-4 levels in gut mucosa during hibernation (shown in previous figure) suggest that functions related to this cytokine, such as secretion of mucosal IgA, are also affected by hibernation. As IgA secretion is critical for defense against invading bacterial pathogens and to maintain the normal intestinal microflora, on goal of these studies was to determine how hibernation affects the body's ability to defend against invading pathogens, despite the long term absence of food intake during the winter months. The results shown below support the conclusion that hibernators do exhibit increased IgA production during the winter fast.



Levels of IgA are generally increased during the hibernation season compared with summer, (EN, entering torpor, LT = late torpor, AR= arousing from torpor, IBA=interbout normothermia). Different letters indicate differences are significant. Asterisk: summer lower than all hibernation states combine. These western blots are supported by immunohistochemistry of gut tissues with anti-IgA antibodies and immunohistochemistry, which shows increased numbers of B cells in the lamina propria during hibernation. IgA molecules appear to be utilized during arousal to protect the gut, then levels are replenished during IBA periods.

Aim 2c: **Determine effect of hibernation on apoptosis in the intestine**. This portion of the project was completed and a manuscript was published (Fleck and Carey, 2006, listed above and attached to email to Dr. Strand). Results showed that there is evidence of accumulation of DNA damage in intestinal epithelial cells during hibernation as the winter progresses. electrophoresis of mucosal DNA revealed no ladders typical of cellular apoptosis. Analysis of changes in pro- and anti-apoptotic signaling pathways in epithelial cells provided evidence for enhanced expression of anti-apoptotic proteins during hibernation that may promote enterocyte survival in a pro-oxidative, pro-apoptotic environment.

Other work not outlined in original proposal but related to overall objective:

Effect of hibernation on pancreatic amylase levels: This award also supported a small study that examined how the exocrine pancreas is affected by hibernation, by determining levels of the digestive enzyme, amylase in pancreatic tissues from summer and hibernating squirrels. Results showed a 40-50% reduction in pancreatic amylase expression during hibernation, which although significant, is not as dramatic as might be expected given the long period of time hibernators are in the fasted state. Thus, maintenance of basal levels of this key digestive enzyme at 50% of summer values despite the extended winter fast likely facilitates the rapid resumption of digestive function after terminal arousal in the spring. This work was published in 2003 (see publication list above).

Protection against intestinal ischemia-reperfusion injury by the hibernation phenotype. Our basic studies carried out at the cellular level (described above) provided evidence for defense mechanisms associated with the hibernation phenotype that protect the gut against the nutritional and physiologic stressors that occur during the hibernation season. The ultimate objective of our studies is to translate hibernation-induced protective mechanisms to improvements in human trauma care, particularly with regard to the warfighter in combat situations. Thus, during the last phase of this project we developed a model of mesenteric ischemia-reperfusion (I/R) injury in ground squirrels, based on similar well-established models in the non-hibernating laboratory rat. The data collection phase of this project has been completed and a paper is in preparation for submission by June, 2006. The key results, outlined below, demonstrate that the hibernator gut is more resistant to gut I/R injury compared with rats or summer squirrels.

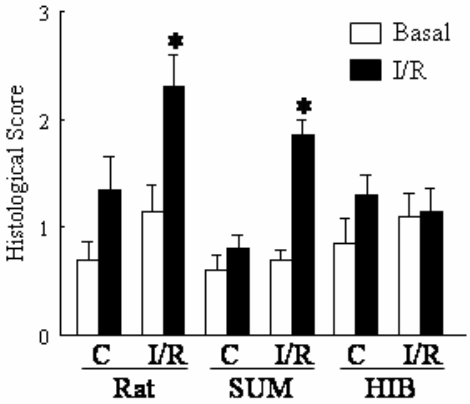
Rationale. Intestinal ischemia-reperfusion injury due to massive HS/R is now recognized as an important early event that leads to local organ dysfunction and ultimately, multi-organ failure (MOF). Mammalian hibernation represent an excellent natural model to study endogenous protective mechanisms to reduce intestinal I/R injury and its systemic complications. The physiological extremes that the gut sustains during torpor-arousal cycles necessitated the development of protective mechanisms to minimize damage to this sensitive organ during the hibernation season. Blood flow to all tissue beds is reduced significantly during torpor, with splanchnic regions, particularly the gut, receiving disproportionately low flow during torpor and arousal relative to other organs like heart, brain, lungs and BAT. It is also one of the last major visceral organs to be fully reperfused upon arousal, which suggests that the gut during hibernation may be subject to moderate degrees of oxidative stress due to regional I/R either during entrance or arousal. We have developed two models of intestinal I/R in rats and ground squirrels: regional ischemia/reperfusion (I/R) of isolated intestinal loops

(**Model I**) and total intestinal I/R (**Model II**). It is important to note that the hibernators used in the studies shown below were aroused to 37 °C T_b when the experiments started, thus hypothermia or hypometabolism at that time the animals were studies is not required for these effects.

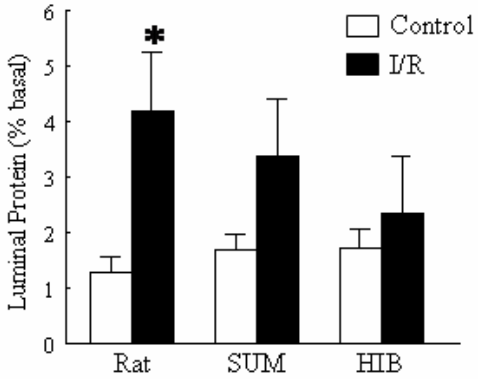
Model I is local I/R injury in response to clamp/release of a branch of the superior mesenteric artery (SMA) that feeds one experimental intestinal loop. An adjacent loop in the same animal that receives continuous blood supply serves as an internal control. At the start of the procedure, the loops were filled with a measured amount of prewarmed PBS for a 30 min control period. At the end of this period, the saline remaining in the loops was collected. The loops were then filled with fresh saline and the arteries leading to the experimental loop were clamped for 20 min and then allowed to reperfuse for 60 min. The control loop remained perfused for the duration of the experiment. At the end of the reperfusion, the remaining saline was collected and the loops were removed and weighed. A 1-cm segment of each loop was fixed in 10% formalin for histological analysis. The remaining loop tissue was opened and remaining saline collected from the loops for measurement of protein content, as an indicator of intestinal permeability changes. *Note all animals were at 37°C body temperature during the experiments,* thus the responses are not dependent on hypothermia per se.

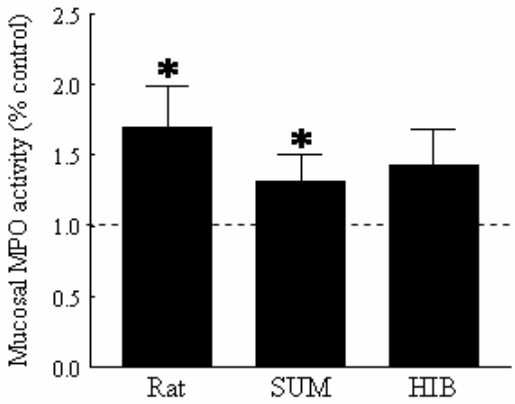
Results of Model I: Local I/R

Histological analysis of intestinal tissue in control loops and after I/R indicates significant damage scores in the I/R loops of rats and summer squirrels, but not aroused hibernators. "Basal" is from section of same loop harvested prior to I/R or control periods. Micrographs of histological damage in rats and summer squirrel I/R loops are very similar to those shown for total intestinal I/R in next section.)



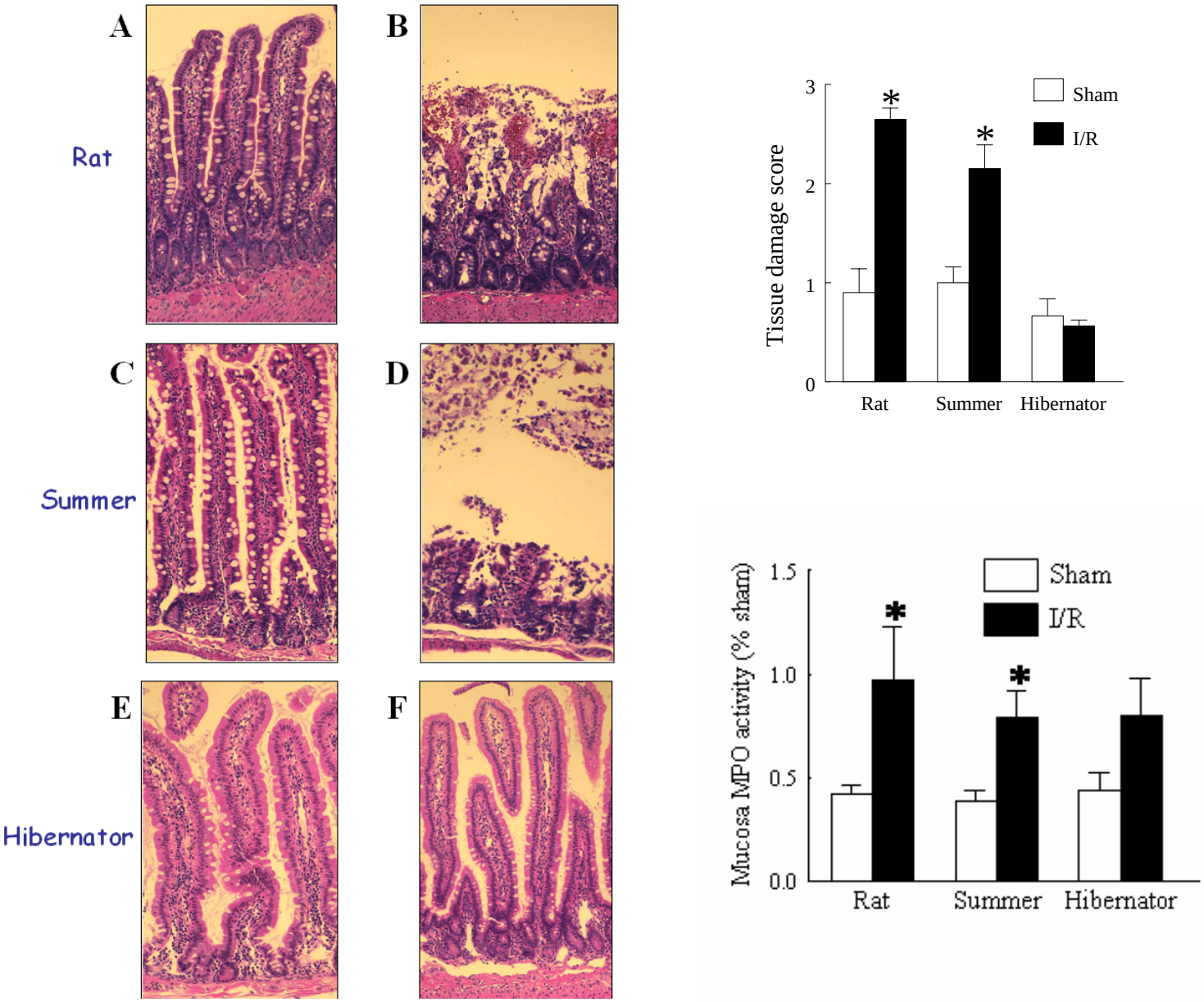
A significant 4-fold increase in protein content was seen in the experimental rat loop following I/R. No significant difference in luminal protein over control was found in summer squirrels despite a 3-fold increase over basal levels. Aroused hibernators were not different from controls. Thus, intestinal permeability increased in rats, showed a strong trend for increase in summer squirrels and did not change in hibernators.



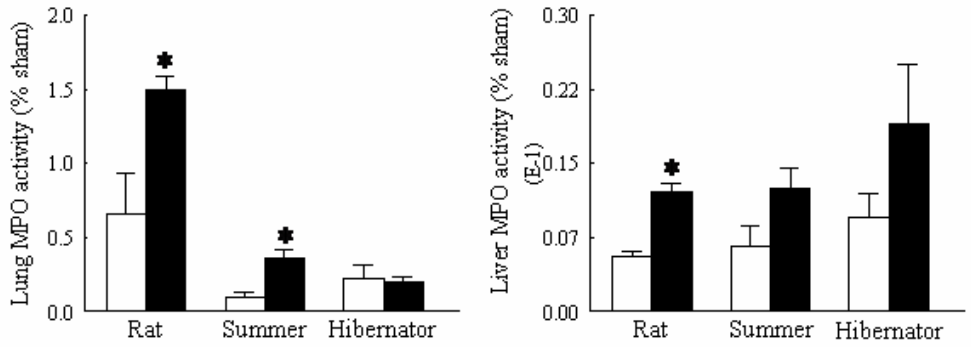


Mucosal MPO activity (reflects neutrophil infiltration, expressed as % of control values) increased in the experimental loop of rats and summer squirrels compared with control, but not in aroused hibernators.

Model II is total SMA occlusion/reperfusion. This model (which was designed and begun during the ARO award but has been completed subsequently via funding from a DARPA award) has been shown to be a clinically relevant model of gut I/R induced shock, which mimics more closely the remote organ effects of uncontrolled hemorrhage compared with controlled hemorrhage as typified by the modified Wiggers model. Similar to Model I, rat intestines compared with aroused hibernators show a higher degree of mucosal damage. *Note all animals were at 37°C body temperature during the experiments.*



Gut tissue is severely damaged by mesenteric I/R in rats and summer squirrels, but not hibernators. Left panel, histology: A,C,E = sham surgery, B, D, F = mesenteric I/R. Upper right, mucosal damage quantitated in histogram, * I/R greater than sham $P<0.05$. Lower right, myeloperoxidase (MPO) as indicator of neutrophil infiltration into mucosa, significant for rats and summer squirrels but not hibernators. N = 4-10 per group.



Changes in neutrophil infiltration (MPO activity) in lungs and liver after mesenteric I/R (solid bars) compared with sham-treated animals (open bars). Changes are significant for rats and summer squirrels in lung and for rats in liver; neither is significant for hibernators. N = 3

As in Model I, intestinal and remote organ damage is most severe in the rats, least in hibernators with summer squirrels intermediate between those groups. Taken together, these results demonstrate that not only are hibernators more resistant to gut damage after I/R, this protection extends to the classic remote organ injury following SMA occlusion shock typically seen in non-hibernators. In this classic response, bacterial translocation (LPS) from the damaged gut mucosal barrier activates hepatic Kupffer cells (liver macrophages) via portal vein delivery. Overwhelmed liver Kupffer cells then spill cytokines into the venous blood directly bathing the pulmonary alveolar macrophages. Activation of the alveolar macrophage promotes synthesis of chemoattractant molecules in the lung (leukotrienes, PAF), which attract circulating neutrophils into the tissue (MPO activity) leading to lung injury and eventually, cardiopulmonary collapse. Our results suggest that the end effect of neutrophil infiltration after SMA occlusion shock is not present in the hibernator. In other studies carried out in a separate project, we found that Kupffer cell activation is increased after cold ischemia-reperfusion injury in rat livers (as expected), and also occurs in livers of summer squirrels, but no activation is seen in livers from hibernating squirrels. This observation provides further support for the conclusion that the inflammatory cascade that is normally initiated after I/R-induced oxidative stress in splanchnic organs is abrogated in hibernators.

In summary, these new studies demonstrate that the intestine during hibernation is better able to withstand a traumatic insult (ischemia-reperfusion) compared with a non-hibernating species (rat). Resistance of summer ground squirrels to these imposed traumas is intermediate between rats and hibernating squirrels, which is expected given that some protection mechanisms may be part of the ability of squirrels to hibernate, and not directly linked to the hibernation phenotype per se. Also important is the dissociation between the protective phenotype we demonstrated in the hibernators from hypothermia per se, because all animals were held at 37°C body temperature in these experimental models. However, the differences we observed between summer and winter squirrels is strong evidence that within the same species, enhanced resistance to stress develops as animals assume the winter hibernation phenotype, in a manner not dependent on profound hypothermia. Thus, our studies initiated during the ARO award suggest that our ultimate goal of identifying mechanisms that induce a protective phenotype in ground squirrels is feasible, and the development of strategies to apply similar types of protection to non-hibernator models of traumatic injury and eventually, humans is within reach.